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Identification of a high affinity NH_4^+ transporter from plants

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Despite the important role of the ammonium ion in metabolism, i.e. as a form of nitrogen that is taken up from the soil by microorganisms and plants, little is known at the molecular level about its transport across biomembranes. Biphasic uptake kinetics have been observed in roots of several plant species. To study such transport processes, a mutant yeast strain that is deficient in two NH_4^+ uptake systems was used to identify a plant NH_4^+ transporter. Expression of an *Arabidopsis* cDNA in the yeast mutant complemented the uptake deficiency. The cDNA *AMT1* contains an open reading frame of 501 amino acids and encodes a highly hydrophobic protein with 9–12 putative membrane spanning regions. Direct uptake measurements show that mutant yeast cells expressing the protein are able to take up [^{14}C]methylamine. Methylamine uptake can be efficiently competed by NH_4^+ but not by K^+. The methylamine uptake is optimal at pH 7 with a K_m of 65 μM and a K_i for NH_4^+ of ~10 μM, is energy-dependent and can be inhibited by protonophores. The plant protein is highly related to an NH_4^+ transporter from yeast (Marini *et al.*, accompanying manuscript). Sequence homologies to genes of bacterial and animal origin indicate that this type of transporter is conserved over a broad range of organisms. Taken together, the data provide strong evidence that a gene for the plant high affinity NH_4^+ uptake has been identified.

Key words: Arabidopsis thaliana/methylamine uptake/nitrogen transport

Introduction

The ammonium ion, which is the most abundant nitrogenous compound apart from N_2, is an important ion involved in nitrogen metabolism and can be transported across the plasma membrane of many organisms. One possible transport mechanism is that ammonia, as a small, uncharged, lipophilic molecule, passively diffuses across the membrane. However, the rates do not seem to be sufficient to explain the flow required for uptake processes.

Carrier-mediated transport of NH_4^+ could be demonstrated in bacteria, fungi and in the kidney of mammals (Hackette *et al.*, 1970; Dubois and Grenson, 1979; Kleiner, 1985; DuBose *et al.*, 1991; Knepper, 1991). In plants, which are strongly dependent on the exogenous nitrogen supply, the uptake of ionic nitrogen from the soil can occur either in the form of nitrate or NH_4^+. Plants living in symbiosis with nitrogen-fixing organisms are additionally able to use atmospheric N_2; the transfer from the symbiotic bacteria into the plant is assumed to occur in the form of ammonia (Glenn and Dilworth, 1984). Ammonium ions are thought to be toxic and therefore are rapidly fixed into amino acids, amides or ureides in the roots. Thus, at least under normal conditions, only very low concentrations of NH_4^+ have been detected in plant cells (Lee and Ratcliffe, 1991). Both low and high affinity NH_4^+ transport has been described for uptake from the soil (Fried *et al.*, 1965; Ullrich *et al.*, 1984; Wang *et al.*, 1993). Uptake systems for NH_4^+ seem to be energy-dependent, as carbon skeletons have to be supplied from photosynthesis in the form of sucrose to the roots plus energy for reduction or to create the electrochemical gradient. In contrast to nitrate uptake, for which a transporter has been isolated by analysis of chlorate-resistant *Arabidopsis* mutants (Tsay *et al.*, 1993), little is known at the molecular level about NH_4^+ transporters in plants or other organisms. It therefore seems important to identify such transport systems to characterize nitrogen uptake processes further. Yeast mutants have proven to be an invaluable tool for identifying genes from homologous and heterologous sources by complementation. By this means, multiple amino acid transport systems have been characterized from *Arabidopsis* (Frommer *et al.*, 1993, 1994; Hsu *et al.*, 1993; Kwart *et al.*, 1993). The approach was also effective for identifying and characterizing several other transport systems from a variety of plants such as K^+ channels and sucrose transporters (Anderson *et al.*, 1992; Riesmeier *et al.*, 1992, 1993; Sentenac *et al.*, 1992). A yeast mutant deficient in two NH_4^+ uptake systems, *MEP1* and *MEP2*, provided a good system for isolating NH_4^+ transporters from plants. These mutations were identified by selecting yeast strains on media containing the toxic NH_4^+ analogue methylamine. A double mutant was constructed that grows only poorly on media containing low concentrations of NH_4^+ as the sole nitrogen source (Dubois and Grenson, 1979). Complementation of the mutant with a cDNA library from *Arabidopsis* seedlings (Minet *et al.*, 1992) has led to the identification of a plant protein that mediates NH_4^+ transport. Characterization of the cDNA, the derived protein and the biochemical properties of this transporter are described. In a parallel study a corresponding gene was isolated from yeast (see accompanying paper by Marini *et al.*, 1994).

Results

Complementation of the NH_4^+ uptake yeast mutations

The *Saccharomyces cerevisiae* strain 26972c, which carries mutations in the two NH_4^+ permease genes, *MEP1* and

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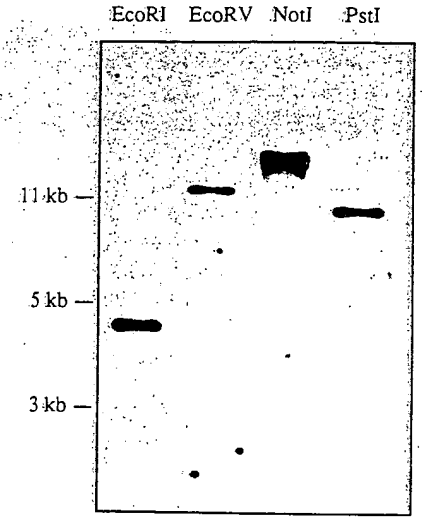


Fig. 1. Southern analysis of the *AMT1* gene. Ten micrograms of genomic DNA isolated from *Arabidopsis* digested with different restriction enzymes were separated by gel electrophoresis, transferred to a nylon membrane and hybridized to the 1.75 kb *AMT1* cDNA.

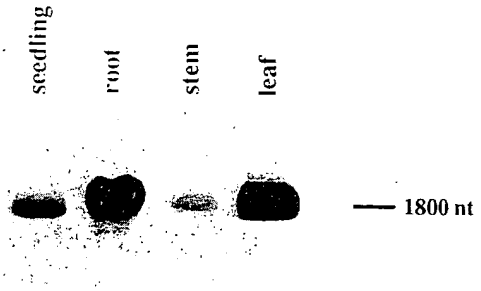


Fig. 2. Expression of the *AMT1* gene in different organs of *Arabidopsis*. Twenty micrograms of total RNA isolated from leaves, stems, roots and seedlings were hybridized to the 1.75 kb *AMT1* cDNA after gel electrophoresis and subsequent transfer of the RNA to nylon membranes.

MEP2, grows very poorly on media containing 1 mM NH_4^+ as the sole nitrogen source. To identify plant NH_4^+ transporter genes, 26972c was transformed with an episomal plasmid containing a cDNA library derived from *Arabidopsis* seedlings under the control of the phosphoglycerate kinase promoter (Minet *et al.*, 1992). Transformants were selected on uracil-free medium, the cells were washed from the plates and an aliquot was plated on medium containing 1 mM NH_4^+ as the sole nitrogen source. A cDNA clone with a size of 1.75 kb (pAMT1) was able to complement the yeast mutation. To confirm that no reversion or second site mutation had occurred in the transformants, the recombinant plasmids were isolated and reintroduced into the mutant strain. Mutant cells transformed with pAMT1 regained the ability to grow on 1 mM NH_4^+ containing minimal medium whereas those transformed with the control plasmid pFL61 did not. This shows that the 1.75 kb cDNA insert from *Arabidopsis* mediates NH_4^+ uptake in yeast. High stringency Southern blot analysis indicates that the *AMT1* gene is present in one or a few copies in the *Arabidopsis* genome (Figure 1). According to Northern analysis the *AMT1* gene is highly expressed as a transcript with a

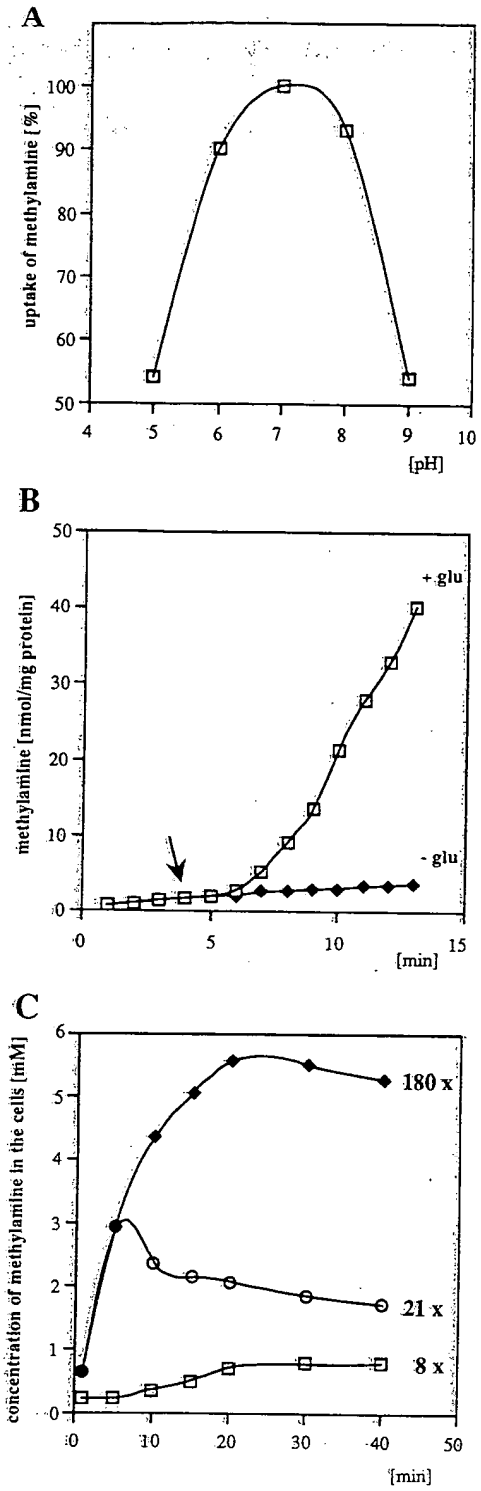


Fig. 3. Methylamine uptake properties of AMT1. (A) pH activity profile for methylamine transport. Uptake was measured under standard conditions in 20 mM sodium phosphate buffer at the pH value indicated. The uptake rate at pH 7 was set to 100% and corresponds to 6 nmol [^{14}C]methylamine/mg protein/min. (B) Dependence of the [^{14}C]methylamine uptake by AMT1 in the presence (+ glu) or absence (− glu) of 100 mM glucose that was added 4 min after the start of the uptake experiment as indicated by the arrow. (C) Accumulation of methylamine in yeast cells expressing AMT1 in the absence of protonophores (◆), with the addition of 2,4-DNP prior to (□) or 5 min after (○) the start of the uptake experiment. The accumulation was determined at pH 5 and otherwise as described in the legend to Table II. The calculated accumulation factors are given to the right.

length of ~1800 nucleotides in roots and leaves of mature soil-grown plants and to lower levels in stems and in seedlings (Figure 2).

Biochemical properties of AMT1

A frequently used method for determining transport kinetics is the measurement of the accumulation of a radioactive substrate. Since no suitable nitrogen isotope is commercially available, [^{14}C]methylamine was used as an NH_4^+ analogue to characterize the AMT1 plant NH_4^+ uptake system in yeast.

To determine optimal conditions for methylamine transport, uptake assays were performed at different pH values. The maximal rate of methylamine uptake by transformed yeast cells was between pH 6 and pH 8, with only half the activity remaining at pH 5 and pH 9 (Figure 3A). To achieve maximal uptake rates, cells were grown in medium containing a high concentration of glucose (2%) and subsequently were incubated with 100 mM glucose before the start of the uptake measurements. Without glucose addition the uptake rate reached only background levels (Figure 3B). This requirement for glucose suggests that the AMT1-mediated uptake is an active process that is dependent on ATP synthesis. This conclusion is supported by the fact that inhibition of *de novo* protein synthesis with cycloheximide 5 min prior to the start of the uptake assay did not strongly inhibit the uptake rate for methylamine (Table I), ruling out the possibility that the carbon source is utilized for the synthesis of proteins directly involved in methylamine transport. Table I shows the influence of protonophores and several inhibitors of ATP synthesis on AMT1 activity. Inhibitors of plasma membrane H^+-ATPase activity such as diethylstilbestrol and DCCD (*N*,*N'*-dicyclohexylcarbodiimide) strongly reduce methylamine uptake. Protonophores such as CCCP (carbonylcyanide *m*-chlorophenylhydrazone) and DNP (2,4-dinitrophenol) that cause the collapse of the electrochemical gradient also block the transport of methylamine.

Since methylamine does not serve as a nitrogen source for yeast (Roon *et al.*, 1975), it was possible to measure the accumulation of this substance simply by determining the intra- and extracellular concentrations of the substrate by measuring the distribution of ^{14}C. In an uptake assay containing an initial methylamine concentration of 100 μM, 26972c yeast mutants expressing AMT1 were able to concentrate methylamine ~700-fold within a 10 min period (Table II). The same strain transformed with the control plasmid pFL61 was capable of concentrating methylamine only 16-fold. This background activity might be due to the presence of a third uptake system for NH_4^+ besides MEP1 and MEP2 (Dubois and Grenson, 1979). The capability to accumulate methylamine against a concentration gradient is strongly reduced when protonophores are present (Figure 3C).

26972c mutants expressing AMT1 take up [^{14}C]methylamine in a saturable, concentration-dependent manner that is consistent with a carrier-mediated uptake. The apparent K_m of this uptake system is ~65 μM when measured in 20 mM sodium phosphate buffer at pH 7. Inhibition studies with NH_4Cl show competitive inhibition with a K_i of 5–10 μM. These values suggest that NH_4^+ is a strong competitive inhibitor of methylamine uptake and that AMT1 represents a high affinity NH_4^+ uptake system (data not shown).

Specificity of the AMT1 transport system

The results of inhibition studies with a variety of potentially competing substances of methylamine uptake are summarized in Table III. With the exception of NH_4^+, which inhibited methylamine uptake by 90%, none of the monovalent cations used in 5-fold excess showed a significant effect. In this context it is of special interest that K^+ obviously does not compete for methylamine uptake even at 5-fold excess. This clearly distinguishes the AMT1 transport system from other plant cation transporters like the K^+ channel KAT1, which has a significant conductivity for NH_4^+ ions (up to 30% of the K^+ conductance; Schachtman *et al.*, 1992). However, in methylamine uptake assays in which the 20 mM sodium phosphate buffer was replaced by 20 mM potassium phosphate buffer, the uptake rate was lowered to 70%. A further increase of the

Table I. Effect of inhibitors on methylamine (MA) uptake

Inhibitor	Relative uptake of MA (%)
None	100
10 μg/ml cycloheximide	85
100 μM diethylstilbestrol	8
20 μM DCCD	34
200 μM DCCD	15
100 μM 2, 4-DNP[a]	18
10 μM CCCP[a]	40

Standard conditions were used. Methylamine was present at a final concentration of 100 μM. The inhibitors were added at a 5-fold molar excess 5 min prior to the reaction start. For each inhibitor the 100% reference value was measured by adding the appropriate volume of solvent without the inhibitor substance. (DCCD, diethylstilbestrol and 2,4-DNP were dissolved in ethanol, and CCCP was dissolved in dimethylformamide).
[a]Assayed at pH 5.

Table II. Accumulation of [^{14}C]methylamine (MA)

Σ26972c transformed with:	Initial MA in the medium (μM)	Final MA concentration		Internal/external
		External (μM)	Internal (μM)	
pFL61	100	66	1000	15
pAMT1	100	4.6	3100	670

...was assayed by adding [^{14}C]methylamine to a cell suspension in 20 mM $NaPO_4$ pH 7 at the given final concentration of MA ...and assayed for relative amounts of [^{14}C]methylamine. Reactions were terminated when ...tions, the cell volume was assumed to be four

potassium buffer concentration up to 100 mM led to a decrease of uptake down to 30% of the value measured in 20 mM sodium phosphate buffer. These data indicate that K^+ only competes for methylamine uptake when present in very high excess (>1000-fold in the case of 100 mM potassium phosphate buffer). The fact that amines with multiple substituents such as dimethylamine, trimethylamine and ethylamine showed no significant inhibitory influence on methylamine uptake rules out the possibility that the AMT1 uptake system is a general transporter of amines rather than a specific NH_4^+ carrier (Table III). Taken together these data confirm that AMT1 represents an NH_4^+ transporter with high specificity.

Sequence analysis of AMT1

DNA sequence analysis of *AMT1* reveals an open reading frame of 501 amino acids which is preceded by a stop codon and has a coding capacity for a protein of 53 kDa. Analysis of the hydrophilicity shows that the predicted protein is highly hydrophobic and contains 9–12 putative membrane spanning regions (Figure 4; Kyte and Doolittle, 1982). No homology to the putative nucleotide binding site (P-loop) of ATP binding proteins involved in primary active transport could be found (Higgins *et al.*, 1990). In contrast to other transporter proteins like the yeast and *Arabidopsis* amino acid permeases (Weber *et al.*, 1988; Kwart *et al.*, 1993) and the homologous protein MEP1 from yeast (Marini *et al.*, 1994), the N-terminus of the AMT1 protein is hydrophobic. Whether this feature indicates the presence of a signal sequence remains to be shown.

A database search for related protein sequences revealed significant homology to three open reading frames with so far unknown functions (Figure 5). One of these putative genes, *nrg*A, is part of the *Bacillus subtilis nrg*29 operon (Atkinson and Fisher, 1991, Wray *et al.*, unpublished: accession no. L03216). The second open reading frame with homology to AMT1 is the 5′ part of a gene of probably bacterial origin named HI. This DNA has been found as a contamination in a rat liver cDNA library (Zheng and Weiner, unpublished: accession no. M98350). The third sequence CEL3 represents a fragment of animal origin and is a translation of a *Caenorhabditis elegans* cDNA clone (Waterston *et al.*, 1992; accession no. M89248). The high homology of these sequences supports the notion that they might also represent NH_4^+ transporters. This conclusion is supported by analyses of the yeast *MEP1* gene (see accompanying paper by Marini *et al.*, 1994). This gene encodes a high affinity NH_4^+ uptake system from *S.cerevisiae* that is also homologous to AMT1 (Figure 5). No homology was found to other ion transporters such as K^+ channels or nitrate transporters.

Table III. Effect of potential competitors on methylamine uptake

Substance	Relative uptake of MA (%)
None	100
Methylamine	40
Dimethylamine	89
Trimethylene	89
Ethylamine	93
KCl	98
RbCl	96
CsCl	98
NH_4Cl	10

Standard conditions were used. Methylamine was present at a final concentration of 100 μM. 100% corresponds to 4 nmol [^{14}C]methylamine/mg protein/min. Competitors were present at a 5-fold molar excess.

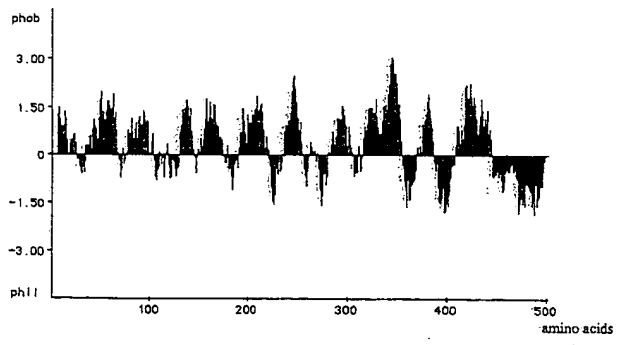


Fig. 4. Hydropathy plot calculated from the amino acid sequence of the NH_4^+ transporter AMT1. The calculation was performed according to Kyte and Doolittle (1982) with a window of 11 amino acids. Hydrophobic regions are given a positive hydropathy index.

Discussion

Plants can take up nitrogen from the soil as either nitrate or NH_4^+. Under most conditions, nitrate is the predominant form of nitrogen in the soil since NH_4^+ produced by mineralization processes or supplied as fertilizer is rapidly converted into nitrate by nitrifying microorganisms. Under field conditions, however, a mixed assimilation of nitrate and NH_4^+ is probably the common situation due to the partial inhibition of nitrification in acidic soils and in agricultural fields. The simultaneous uptake of both nitrogen sources also reduces the problem of pH stress for the plant, since the by-products (H^+ and OH^-) of NH_4^+ and nitrate assimilation can neutralize each other. After the uptake of NH_4^+ into the root, it is either transported in the form of ammonium malate or is readily fixed into amino acids which are translocated through the vascular system to the shoot (Raven and Smith, 1976). In both cases the charge balance would be maintained, since the influx of positively charged NH_4^+ ions could readily be neutralized by exporting an equivalent amount of protons directly to the soil.

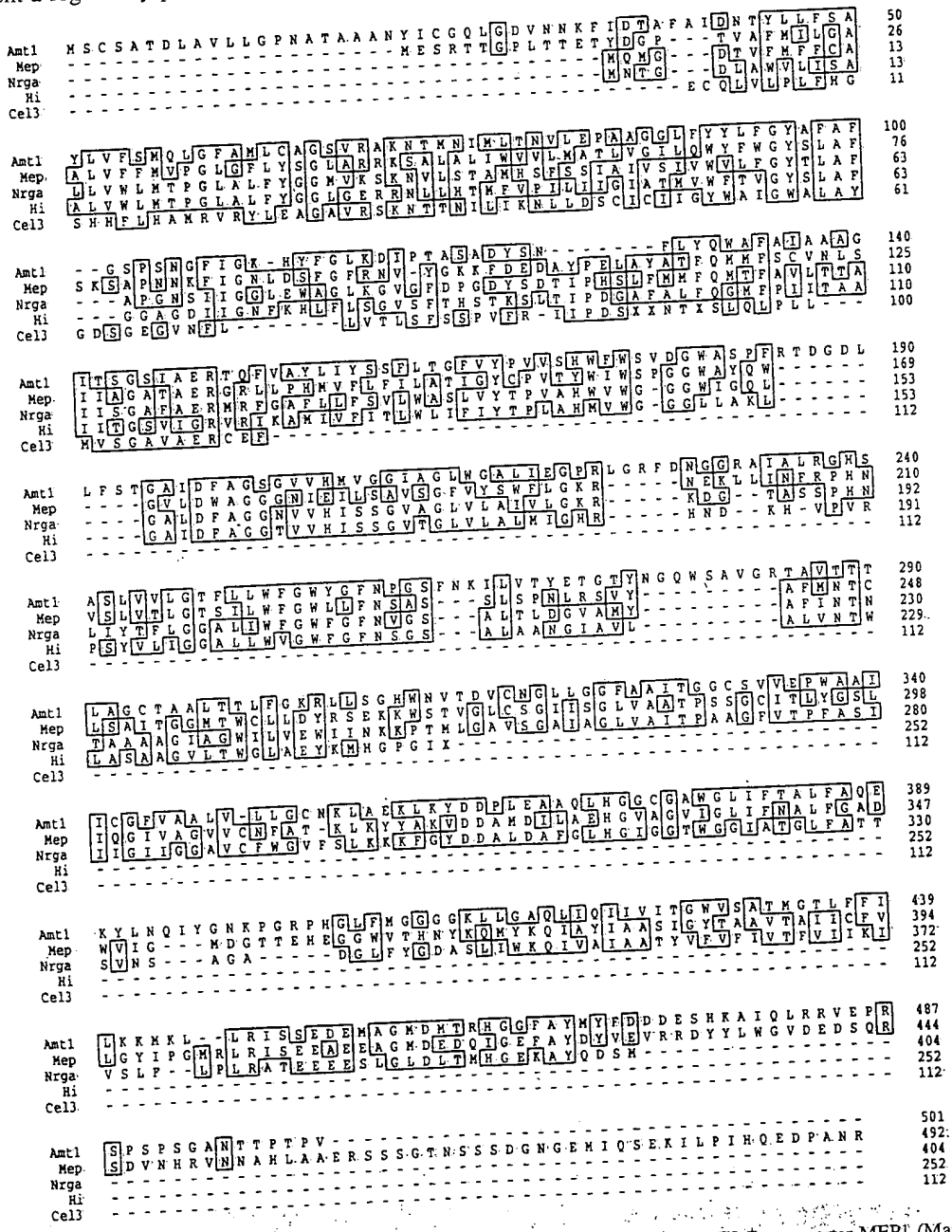
Although numerous NH_4^+ carriers have recently been described genetically or physiologically, the molecular basis for such a transport remains unclear and is a matter of debate. For many years it was accepted that NH_3 as a small uncharged lipophilic molecule can pass the plasma membrane without the necessity for specific uptake systems. The identification of mutants for NH_3 uptake in multiple species has challenged this model (Kleiner, 1985). This and the fact that at neutral and acidic pH most ammonia is protonated and in this form not able to pass the membrane has led to the proposition of specific carriers for NH_4^+. In unicellular organisms like bacteria and yeast an important role for such transport systems might be to counteract the passive efflux of NH_3 as retrieval systems (Kleiner, 1985; Peña *et al.*, 1987).

Molecular approaches for isolating NH_4^+ transporter genes

Recently transporters for nitrate and K^+ could be identified (Anderson *et al.*, 1992; Sentenac *et al.*, 1992; Tsay *et al.*, 1993). In contrast, very little is known about the molecular basis of NH_4^+ transport. Attempts to isolate NH_4^+ transport systems by complementation of NH_4^+ uptake mutants from a variety of species have not been successful. These attempts allowed the identification of several genes which most likely do not code for carrier proteins. The *Escherichia coli amtA* gene seems to encode a cytoplasmic component of the NH_4^+ transport system (Fabiny *et al.*, 1991). The *Rhodobacter adgA* gene which complements a mutation of a starvation-inducible NH_4^+ uptake system seems to represent a regulatory protein (Zinchenko *et al.*, 1990). The same *Rhodobacter adgA* mutation can be complemented by the *E.coli* gene *efg*, whose function is unknown (Willison, 1993).

Expression of AMT1 in yeast

Kinetic studies indicate the presence of at least three NH_4^+ transport systems in *S.cerevisiae*. Two of these uptake systems, MEP1 and MEP2, are kinetically well characterized, whereas a third uptake system has been postulated based on the remaining uptake activity in a *mep1mep2* double mutant (Dubois and Grenson, 1979). The MEP1 uptake system is a high affinity and high capacity transporter (K_m: 2 mM), whereas MEP2 has an even higher affinity but lower capacity to transport the NH_4^+ analogue methylamine (K_m: 250 μM). These trans-



...id sequence alignment of the plant NH_4^+ transporter AMT1 (this work), the yeast NH_4^+ transporter MEP1 (Marini *et al.*, 1994) ... unknown origin (Hi) and *Caenorhabditis elegans* (Cel3).

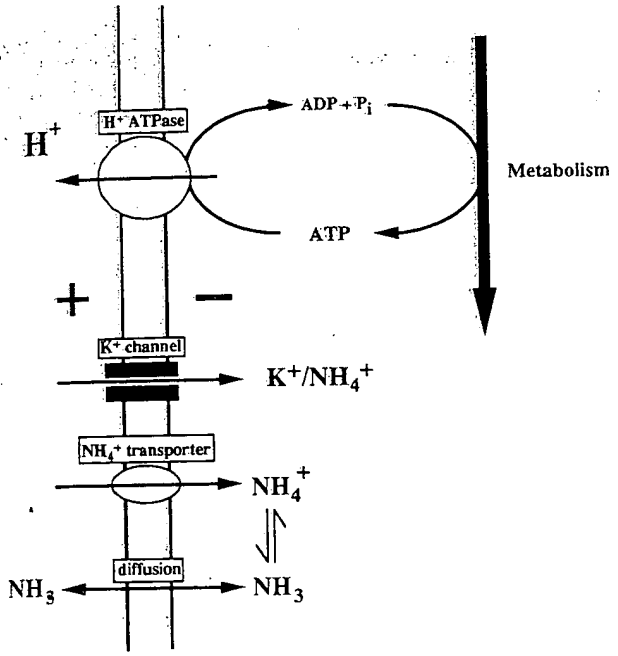


Fig. 6. Possible mechanisms for NH_4^+ uptake. The H^+-ATPase, linked to metabolism, generates the membrane potential that drives NH_4^+ inside the cell. Besides carrier molecules with high affinity and specificity like AMT1, K^+ channels can mediate NH_4^+ uptake. At neutral or acidic pH, passive diffusion of ammonia contributes only marginally to the transport.

port functions are inhibited by NH_4^+ with K_i values of 20 μM (MEP1) and 1 μM (MEP2). The double mutant grows only poorly on 1 mM NH_4^+ as the sole source of nitrogen. Complementation of this mutant has proven to be an effective system for isolating and characterizing both homologous and heterologous NH_4^+ transporter genes. The data summarized below demonstrate the existence of an *Arabidopsis* NH_4^+ transport system which with regard to its biochemical properties and protein sequence resembles high affinity NH_4^+ uptake systems from *S.cerevisiae*.

Both methylamine transport in yeast (Roon *et al.*, 1975) and AMT1-mediated uptake show a pH optimum in the neutral range. This activity profile suggests that methylamine (pK_a 10.6) as well as NH_4^+ (pK_a 9.2) are transported or initially bound by the transporter in their protonated form. Furthermore, this pH optimum distinguishes it from other *Arabidopsis* transporters such as amino acid and sucrose carrier proteins that show highest activities at low external pH, a property which agrees with their predicted role as proton cotransporters.

Regarding its high affinity for methylamine (K_m: 65 μM) and the low K_i value for inhibition by NH_4^+ (<10 μM), AMT1 seems to be equivalent to the high affinity NH_4^+ uptake systems of yeast. This finding is supported by the fact that AMT1 and MEP1 show strong homology on the protein level.

Both wild type yeast cells and the plant transporter expressed in the *mep1mep2* double mutant are capable of concentrating methylamine several hundred-fold against a concentration gradient, a process that is indicative of an active transport mechanism. For efficient uptake a studies with *S.cerevisiae* support the view that the energy for NH_4^+ transport is provided by ATP through the operation of a plasma membrane H^+-ATPase (Peña *et al.*, 1987).

Mechanism of NH_4^+ uptake

The sensitivity of AMT1 activity in yeast towards protonophores and its dependence on an energy source and ATP synthesis can be taken as indications that this system transports along the electrochemical gradient and is dependent on the plasma membrane ATPase activity. The H^+-ATPase activity would allow the cell to compensate for the depolarization of the membrane potential caused by the influx of the positively charged NH_4^+ ions. Since depolarization of the membrane will stimulate the H^+-ATPase and thus lead to a high turnover of ATP the energy dependence of the uptake system can be explained (Figure 6). Such a model of a secondary active uptake system has also been proposed for yeast NH_4^+ transport (Peña *et al.*, 1987). Since the characteristics of NH_4^+ uptake resemble those of K^+ uptake in yeast, it has been speculated that NH_4^+ and K^+ are transported by the same general mechanism but different carriers (Peña *et al.*, 1987). For the transport of K^+ into plant cells several authors (e.g. Behl and Raschke, 1987) propose that plasmalemma K^+ influx is coupled to the active extrusion of protons. Recently *Arabidopsis* K^+ channels (KAT1 and AKT1) have been isolated by complementation of K^+-transport-deficient yeast mutants (Anderson *et al.*, 1992; Sentenac *et al.*, 1992). Electrophysiological analysis allowed characterization of KAT1 as an inwardly-rectifying K^+ channel (Schachtman *et al.*, 1992), suggesting that K^+ influx might be driven by the negative membrane potential of the cells. However, the precise mechanism of K^+ uptake into plant cells remains a matter of debate. Calculations of the electrochemical potential for K^+ in the cytoplasm versus the solution at the root surface suggest that the electrochemical potential gradient for K^+ could not facilitate K^+ uptake from a solution containing K^+ in the micromolar range under physiological conditions (Kochian and Lucas, 1993). A similar conclusion was drawn from a thermodynamic analysis of K^+ absorption into *Arabidopsis* roots (Maathuis and Sanders, 1993). This analysis suggests that, due to the high cytosolic K^+ concentration found in the plant cell, the driving force for K^+ will be outwardly directed and thus K^+ uptake cannot be solely energized by the proton motive force. In case of NH_4^+ uptake the situation seems to be much simpler. The fixation of NH_4^+ into amino acids by metabolic processes results in the intracellular levels of NH_4^+ being low enough to allow channel-mediated uptake driven by the internal negative membrane electrical potential. However, the present data on AMT1 cannot rule out alternative transport mechanisms such as symport or antiport with H^+ or other ions and it may even be possible that NH_4^+ is actively accumulated into the vacuole by AMT1 and that transport into the cell simply follows a concentration gradient. Electrophysiological studies are required to unambiguously demonstrate the transport mechanism.

Specificity of the NH_4^+ transporter

The AMT1 uptake system is strongly inhibited by NH_4^+ but only slightly by K^+ and other monovalent cations (Table III). This high specificity for NH_4^+ clearly distinguishes AMT1 from plant K^+ channels like KAT1 which has been shown to transport NH_4^+ with about one-third of its capacity for K^+ (Schachtman *et al.*, 1992). Electrophysiological studies have provided evidence for cross-competition of K^+ and NH_4^+ for both the low and high affinity transport systems (Vale *et al.*, 1988; Henriksen *et al.*, 1992). In many cases, however, the analysis at the whole plant and plasma membrane level is complicated by the presence of multiple transport systems that cannot be resolved by kinetic studies. The characteristics of AMT1 indicate the existence of a new class of transporters that are highly specific for the transport of NH_4^+.

Sequence analysis and homologies to other proteins

Analysis of the hydrophobicity predicts that 9–12 hydrophobic segments are present in AMT1 (Figure 4). Such a hydrophobicity pattern is characteristic of a variety of integral membrane transporter proteins belonging to the class of single transport proteins that mediate both substrate recognition and translocation. The absence of a putative ATP binding site suggests that the energy required for active transport of NH_4^+ is not acquired by direct hydrolysis of ATP and indicates that AMT1 is a secondary transport protein. AMT1 is homologous to the yeast counterpart (53% similarity). An interesting difference between AMT1 and the yeast MEP1 protein is the presence of a 21 amino acid long hydrophobic domain at the N-terminus of the plant transporter protein which might be involved in plasma membrane targeting. No such leader sequences were found in other transporters such as the sucrose or amino acid transporters (Riesmeier *et al.*, 1992, 1993; Kwart *et al.*, 1993). A search for other homologous sequences led to three yet uncharacterized putative genes. Alignment of the four protein sequences from different kingdoms (Figure 5) shows significant homologies and may indicate that the AMT1 type NH_4^+ carrier is evolutionarily conserved in bacteria, fungi, plants and animals.

Regulation of NH_4^+ transport

In yeast both MEP1 and MEP2 transport activities are strongly repressed in cells grown in the presence of NH_4^+ and thus are subject to nitrogen catabolite repression. There is evidence that this repression mechanism acts at the level of permease synthesis and not on the carrier protein itself (Dubois and Grenson, 1979). Preliminary results show that AMT1 uptake activity indeed is not lowered in cells grown in the presence of NH_4^+ (data not shown). To obtain further information about the regulation of *AMT1* it will be interesting either to analyse the gene with its own transcriptional control region in yeast or to assay *AMT1* expression directly in plants grown under various nutritional conditions.

Function in the plant and outlook

The expression of *AMT1* in roots and the similarity of its properties to the high affinity root uptake system from [illegible] 1993) strongly support a function in the uptake of NH_4^+ from the soil. Such a function would be analogous to that of the related yeast MEP system which is responsible for uptake of NH_4^+ from the environment. However, currently we cannot exclude the possibility that the protein is involved in retrieval of NH_3 that has leaked out of the cells by diffusion—a function suggested for the bacterial transporters (Kleiner, 1985). The expression of *AMT1* in leaves and in the stem might be an indication for such a role of the transporter besides uptake of NH_4^+ from the soil. Under stringent conditions one gene could be detected in Southern analysis. However, this does not rule out the possibility of the presence of further more distantly related genes. Analysis of regulation and *in situ* localization of *AMT1*, e.g. by analysing differences in expression in the presence of different nitrogen sources, may help to unravel the actual function of this protein. These studies will be combined with use of the *AMT1* gene to identify related genes. Furthermore, expression of the gene in the antisense orientation in the plant will possibly be a useful tool to analyse role and function in the plant as has been shown for the sucrose transporter (Riesmeier *et al.*, 1993, 1994). The availability of such genes in the future might enable the manipulation of the transport characteristics of different organs and the uptake characteristics for nitrogen in transgenic plants.

Materials and methods

Strains and plant material

The yeast strain 26972c (*mep1 mep2 ura3*) was constructed by introducing a *ura3* deficiency into strain 01220b (Dubois and Grenson, 1979; B.André, Bruxelles, personal communication). The plant material was *Arabidopsis thaliana* L. Heynh., ecotype C24.

Yeast growth, transformation and selection

The yeast strain 26972c was transformed with a cDNA expression library derived from *Arabidopsis* seedlings in the yeast expression vector pFL61 (Dohmen *et al.*, 1991; Minet *et al.*, 1992). Transformants were selected on SD medium without uracil, washed from the plates in nitrogen-free medium (Difco, Augsburg) and selected on nitrogen-free medium supplemented with 10 mg/ml glucose and 1 mM ammonium sulfate as the sole nitrogen source. Colonies able to grow were selected in liquid medium, and plasmid DNA was isolated and reintroduced into the mutant 26972c to demonstrate that the ability to grow was dependent on the presence of the plant cDNA.

DNA manipulations and sequence analysis

The *AMT1* cDNA was excised from the yeast expression vector and subcloned. Both strands were sequenced with T7 polymerase (Pharmacia, Freiburg) from a set of deletions and by using synthetic oligonucleotides. The DNA sequence analysis was performed using the UWGCG package (Devereux *et al.*, 1984) and has been submitted to the EMBL DataBank under the accession number X75879. The search for related protein sequences was done using the BLAST program (Altschul *et al.*, 1990) for screening of the NCBI databank. Isolation of genomic DNA from leaf material was performed according to Rogers and Bendich (1985) and RNA was isolated from different organs of *Arabidopsis* as described by Logemann *et al.* (1988). Southern and Northern hybridizations were performed at 68°C in a buffer containing 7% SDS, 1 mM EDTA, 1% BSA and 250 mM sodium phosphate, pH 7.2. The 1.75 kb *Not*I fragment from pAMT1 was used as a probe. Filters were washed at 68°C three times in 2 × SSC, 0.1% SDS.

Transport measurements

For standard uptake studies, yeast cells were grown to the logarithmic phase in NAAG medium [2% glucose, 1.7 g/l nitrogen base without NH_4^+ and amino acids (Difco)] supplemented with 500 μg/ml L-proline. Cells were harvested at an OD_{660} of 0.5–0.7, washed and resuspended in 20 mM phosphate buffer, pH 7, to a final OD_{660} of 8. Prior to the

uptake measurements, the cells were supplemented with 100 mM glucose and incubated for 5 min at 30°C. To start the reaction, 100 µl of this cell suspension were added to 100 µl of the same buffer containing 18.5 kBq of [^{14}C]methylamine (NEN) and different concentrations of unlabelled methylamine (Sigma) and inhibitors depending on the experiment. The standard assay contained 100 µM methylamine. Samples (50 µl) were taken after 10, 60, 120 and 180 s, transferred to 4 ml of ice-cold water, filtered on glass fibre filters, and washed with 8 ml of water. To reduce non-specific binding, the washing solution was supplemented with 5 mM methylamine. The uptake of ^{14}C was determined by liquid scintillation spectrometry (Beckman).

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APENDIX A

Apendix A shows a comparison of the amino acid sequences of the spybean ammonium transporter (SEQ ID NO: 4) and the Ammonium transporter from *Arabidopsis thaliana* (gi: 1703292). Identical amino acids are indicated with an asterix above the alignment. Dashes are used by the program to maximize alignment of the sequences.

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                **   * *  ** ***** *   ***  ***    *  ** ***** *************
SEQ ID NO:4     MSLPACPAEQLAQLLGPNTTDASAAASLICGHFAAVDSKFVDTAFAVDNTYLLFSAYLVF
Gi:_1703292     MS---CSATDLAVLLGPNAT---AAANYICGQLGDVNNKFIDTAFAIDNTYLLFSAYLVF

                ****************************************** ************** **
SEQ ID NO:4     SMQLGFAMLCAGSVRAKNTMNIMLTNVLDAAAGGLFYYLFGFAFAFGSPSNGFIGKHFFG
Gi:_1703292     SMQLGFAMLCAGSVRAKNTMNIMLTNVLDAAAGGLFYYLFGYAFAFGSPSNGFIGKHYFG

                *****  * *** ************************************************
SEQ ID NO:4     LKDIPSSSYDYSYFLYQWAFAIAAAGITSGSIAERTQFVAYLIYSSFLTGFVYPVVSHWF
Gi:_1703292     LKDIPTASADYSNFLYQWAFAIAAAGITSGSIAERTQFVAYLIYSSFLTGFVYPVVSHWF

                ** *****      * ***** ***************************** ****  ***
SEQ ID NO:4     WSPDGWASAFKIT-DRLFSTGVIDFAGSGVVHMVGGIAGLWGALIEGPRMGRFDHAGRAV
Gi:_1703292     WSVDGWASPFRTDGDLLFSTGAIDFAGSGVVHMVGGIAGLWGALIEGPRLGRFDNGGRAI

                ********************************** **   * * *****************
SEQ ID NO:4     ALRGHSASLVVLGTFLLWFGWYGFNPGSFNKILLTYGNSGNYYGQWSAVGRTAVTTTLAG
Gi:_1703292     ALRGHSASLVVLGTFLLWFGWYGFNPGSFNKILVTY-ETGTYNGQWSAVGRTAVTTTLAG

                 ***********  ********************** *********** *****  **  *
SEQ ID NO:4     STAALTTLFGKRVISGHWNVTDVCNGLLGGFAAITAGCSVVEPWAAIVCGFVASIVLIAC
Gi:_1703292     CTAALTTLFGKRLLSGHWNVTDVCNGLLGGFAAITGGCSVVEPWAAIICGFVAALVLLGC

                ****** * ************** ** *******   *    **   ** *** ******
SEQ ID NO:4     NKLAEKVKFDDPLEAAQLHGGCGTWGVIFTALFAKKEYVKEVYGL--GRAHGLLMGGGGK
Gi:_1703292     NKLAEKLKYDDPLEAAQLHGGCGAWGLIFTALFAQEKYLNQIYGNKPGRPHGLFMGGGGK

                ** *  *** ** ******** **  * * ********** ************* * **
SEQ ID NO:4     LLAAHVIQILVIAGWVSATMGPLFWGLNKLKLLRISSEDELAGMDMTRHGGFAYAYEDDE
Gi:_1703292     LLGAQLIQIIVITGWVSATMGTLFFILKKMKLLRISSEDEMAGMDMTRHGGFAYMYFDDD

                      ***** *   *
SEQ ID NO:4     THKHGMQLRRVGPNASSTPTTDE-----
Gi:_1703292     ESHKAIQLRRVEPRSPSPSGANTTPTPV
```